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13. ABSTRACT (Maximum 200 Words) The p53 gene mediates a major tumor suppression pathway that is frequently altered in human tumors, including breast cancer. The p53 protein presents in low amounts in normally growing cells and is activated in response to physiological insults. MDM2 regulates p53 either through inhibiting p53's transactivating function in the nucleus or by targeting p53 degradation in the cytoplasm. We identified a novel nuclear export signal (NES) in the NH ₂ -terminus of p53, spanning residues 11 to 27 and containing two serine residues phosphorylated following DNA damage, which was required for p53 nuclear export in collaboration with the COOH-terminal NES. Ser ¹⁵ -phosphorylated p53 induced by UV irradiation was not exported. Thus, DNA damage-induced phosphorylation may achieve optimal p53 activation through inhibiting both MDM2 binding to and the nuclear export of p53. We have previously discovered that ARF, a tumor suppressor that is induced by various oncogenic insults and functions to activates p53, blocks p53 degradation by MDM2 via inhibiting p53 nuclear export. Our current finding expands the role of nuclear export in controlling p53.				
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NOVEMBER 19, 2002**RE: ANNUAL REPORT FOR DAMD 17-99-1-9574****PI: YUE XIONG, PH.D.****INTRODUCTION**

The p53 tumor suppressor gene mediates a major tumor suppression pathway in mammalian cells that is frequently altered in human cancers (Levine, 1997; Prives, 1998). p53 protein is kept at low level during normal cell growth by its short half-life and is stabilized following various cellular insults. Growing evidence has identified MDM2 as a key regulator of p53 protein stability. Both MDM2 and its human homolog, HDM2, can bind to and abrogate p53 function either by repressing p53's transcriptional activity in the nucleus (Momand et al., 1992; Oliner et al., 1993; Thut et al., 1997), or by targeting p53 for degradation in the cytoplasm (Haupt et al., 1997; Kubbutat et al., 1997), reviewed in (Zhang and Xiong, 2001a). Deletion of the MDM2 gene in mice results in early embryonic lethality, which can be rescued by the simultaneous deletion of p53, supporting the notion that p53 is the major target of MDM2 during development (Jones et al., 1995; Luna et al., 1995).

Blocking p53 nuclear exporting leads to p53 stabilization (Roth et al., 1998), indicating that p53 degradation occurs in the cytoplasm and suggesting p53 nuclear export as a major regulatory event in MDM2-mediated p53 degradation (Zhang and Xiong, 2001a). Two best characterized pathways leading to p53 stabilization and activation are oncogenic stimulation that activates the expression of ARF tumor suppressor gene and DNA damage which activates various protein kinases and causes phosphorylation of both p53 and MDM2 proteins. We previously reported that ARF inhibits p53 degradation in part by blocking p53 nuclear export (Zhang and Xiong, 1999). We also found that the N-terminal region of p53 contains a functional NES (nuclear export sequence) that is required for p53 nuclear export. We further demonstrated that this N-terminally situated NES contains at least one residue, Ser-15, that is phosphorylated following DNA damage and that phosphorylation at Ser-15 inhibited p53 nuclear export (Zhang and Xiong, 2001b).

Mdm2 was first cloned as an amplified gene on a murine double-minute chromosome (Fakharzadeh et al., 1991) and was subsequently found to be amplified in a portion of human sarcomas (Oliner et al., 1992) and brain tumors (Corvi et al., 1995; Reifemberger et al., 1993). In addition to genomic amplification and overexpression of HDM2, various mutations within the HDM2 gene have been reported in several types of cancers (Schlott et al., 1997). The prevalence of HDM2 mutations in human tumors is not clear and the functional significance of these mutations has not been characterized. Over the past year, we have made significant progress in understanding the physiological significance and biochemical mechanism underlying these tumor-associated mutations in MDM2.

KEY RESEARCH ACCOMPLISHMENT

Tumor-derived mutations in the central zinc finger domain of HDM2 disrupt its association with ribosomal proteins L5 and L11

To determine the functional consequence of mutations or deletions of the central zinc finger region, we examined the protein complex formation of these HDM2 mutants with two known binding proteins, p53 and ARF. Wild-type and mutant HDM2 were ectopically expressed in HeLa cells in the presence or absence of p53 and ARF. Protein complex formation was determined by coupled metabolic labeling and immunoprecipitation. Neither point mutations at Cys305 (HDM2^{C305F}) or Cys308 (HDM2^{C308Y}), nor truncations at residue 302 (HDM2¹⁻³⁰²) or 309 (HDM2¹⁻³⁰⁹) had any detectable effect on the ability of HDM2 to bind with p53 or ARF. Comparison of wild-type and mutant HDM2 complexes led to the identification of binding of ribosomal protein L11 with HDM2.

Zinc finger mutant HDM2 is more active in repressing p53's transactivating function

We determined the effect of zinc finger mutations on the ability of HDM2 to repress p53 transactivation function using the p53-responsive reporter construct. Co-expression of wild-type HDM2 and HDM2^{C305F} decreased luciferase activity in a dose-dependent manner. In various concentrations compared, mutant HDM2^{C305F} consistently exhibited a more pronounced inhibition of p53 transcriptional activity than did wild-type HDM2. These results demonstrate that mutations in the zinc finger motif, while destroying the ability of HDM2 to degrade p53, does not abolish, and may even enhance, HDM2's ability to repress p53-mediated transactivation.

L11 reduces HDM2-mediated p53 ubiquitination and activates p53

To determine how L11 affects the activity of both HDM2 and p53, we examined whether L11 interferes with HDM2-mediated p53 ubiquitination *in vivo*. Co-expression of L11 with HDM2 and p53, like that of ARF, almost completely blocked the accumulation of polyubiquitinated p53. Consistent with the decrease of p53 ubiquitination, L11 prevents HDM2-mediated p53 degradation, resulting in an increase of the steady state level of p53 protein in the presence of HDM2. Ectopic expression of L11 resulted in a dose-dependent increase of the steady state level of endogenous p53 protein in U2OS cells, while overexpression of p53 did not appear to significantly affect L11 protein level. These results demonstrate that L11 protein interferes with HDM2-mediated p53 ubiquitination and subsequent degradation.

We then determined the effect of L11 on HDM2-mediated repression of p53 transactivation activity. Under the condition where HDM2 almost completely repressed p53-dependent transactivation from the pGL3-Luc reporter, co-transfection with an L11 expression plasmid restored up to 70% of p53 transactivation activity, but not HDM2^{C305F}-imposed p53 repression.

Ectopic expression of L11 blocks S phase entry in a p53-dependent manner

The ability of L11 to block HDM2-mediated repression and degradation of p53 predicts that L11 could reverse HDM2 inhibition of p53-mediated cell cycle arrest. Indeed, we found that cotransfection with L11 in cells overexpressing p53 and HDM2 restored the S-phase entry block to a level comparable to that seen in cells co-expressing ARF. Also reminiscent of ARF, singular transfection of L11 blocked S-phase entry in p53 wild-type U2OS cells but had little effect on the S-phase entry of p53-deficient SAOS-2 cells. This result is consistent with the idea that L11 blocks S-phase entry with a correlative dependence on p53 function.

Perturbation of ribosomal biogenesis increases L11-HDM2 complex and p53 level

We tested the idea that the L11-HDM2-p53 pathway may function in monitoring ribosomal biogenesis and cell growth. We treated cells with a low concentration of actinomycin D, a polypeptide-containing antibiotic that binds to DNA and inhibits selectively inhibit RNA pol I-dependent transcription and thus ribosomal biogenesis at low concentration. In untreated cells endogenous L11 localized to the nucleolus and the cytoplasm, consistent with the nucleolus as the site for ribosomal subunit assembly and the cytoplasm for the final localization of mature ribosomes. After treating cells with a low concentration of actinomycin D, L11 was no longer seen in the nucleolus while the intensity of cytoplasmic L11 signals appeared unchanged. There was a slight increase of the steady state level of L11 protein following actinomycin D treatment, while the level of HDM2 was increased by three-fold. Associated with this increase of HDM2 levels was an increase of L11-HDM2 complex formation and a corresponding increase in p53 protein by 1.5 fold.

REPORTABLE OUTCOMES

These results have resulted in a manuscript that has been submitted for publication.

CONCLUSIONS

The MDM2 oncoprotein negatively regulates the p53 tumor suppressor by either direct repression of p53 transactivation activity in the nucleus or promotion of p53 degradation in the cytoplasm. In certain types of human cancers such as sarcomas and brain tumors, MDM2 gene amplification is a common event and contributes to p53 functional inactivation. We have found that the human homologue of MDM2, HDM2, binds to ribosomal protein L11 and that the L11-HDM2 association is disrupted by mutations derived from several types of human tumors that target the central zinc finger of HDM2. L11 prevents HDM2-mediated p53 ubiquitination and degradation, restoring p53-mediated transactivation and cell cycle arrest. Interference of ribosomal biogenesis increased the amount of L11-HDM2 complex and the level of p53. Our finding that tumor-derived mutations targeting the central zinc finger disrupt a negative regulation of HDM2 represents a novel mechanism of HDM2 oncogenic activation. These results suggest a function for a L11-HDM2-p53 pathway in monitoring cell growth and a novel mechanism for oncogenic activation of HDM2.

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APPENDICES

None

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Statement of Work

We initially proposed an investigation on "G1 Cell Cycle Control in Normal and Tumorigenic Breast Cells by Regulated Proteolysis". Specifically, we proposed to test the idea that a newly discovered family E3 ubiquitin ligases, the ROC-cullin ligases (ROC was formally known as CUR), may interact with regulate cyclin D, a key G1 cell cycle regulator that is amplified in a subset of breast tumors, regulate the ubiquitination, and thus degradation and the steady state level of cyclin D1 protein. While the study on the ROC-cullin ligase is still in progress, we have not yet determined how ROC-cullin ligase control the ubiquitination of cyclin D1. In a separate development directly related to the suppression of breast cancer and other type of human tumors, we have made a significant and unexpected discovery. This finding, as described in detail in the annual report, concerns the control of ubiquitination and degradation of p53, a tumor suppressor and G1 regulator that is frequently mutated in wide range of human tumors, including breast cancer. The p53 protein presents in low amounts in normally growing cells and is activated in response to physiological insults. MDM2 regulates p53 either through inhibiting p53's transactivating function in the nucleus or by targeting p53 degradation in the cytoplasm. We identified a novel nuclear export signal (NES) in the N-terminus of p53 that is required for p53 nuclear export. We have found that DNA damage-induced phosphorylated in this NES inhibited p53 nuclear export, whereby, blocking the cytoplasmic degradation of p53 and leading to p53 accumulation and functional activation in the nucleus.

We have previously discovered that ARF, a tumor suppressor that is induced by various oncogenic insults and functions to activates p53, blocks p53 degradation by MDM2 via inhibiting p53 nuclear export. Our current finding expands the role of nuclear export in controlling p53. We are continuously to investigate the function of ROC-cullin ubiquitin ligases in normal and tumorigenic breast cells. Our future studies will include the control of p53 ubiquitination.